EAST Search History

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
		"peptide 39" and $ethylcarbamoyl$	US-PGPUB; USPAT	OR	ON	2006/05/23 18:27
S1	1	("20030215788").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2006/05/22 21:24
S2	1354655	peptide (protect$ OR deprotect$) cyst$5	US-PGPUB; USPAT	OR	ON	2006/05/22 18:34
S3	656	peptide (protect$ OR deprotect$) cyst$5	US-PGPUB; USPAT	WITH	ON	2006/05/22 18:34
S4	656	peptide (protect$ OR deprotect$) cyst$5	US-PGPUB; USPAT	WITH	ON	2006/05/22 18:34
S5	2	peptide (protect$ OR deprotect$) cyst$5 immobil$	US-PGPUB; USPAT	WITH	ON	2006/05/22 18:44
S6	145	blake-james$.inv.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	WITH	ON	2006/05/22 18:48
S7	10	cole-carol$.inv.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	WITH	ON	2006/05/22 18:46
S8	72	coleman-patrick$.inv.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	WITH	ON	2006/05/22 18:46
S9	12	monji-nobuo$.inv.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	WITH	ON	2006/05/22 18:45
S10	128	montana-john$.inv.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	WITH	ON	2006/05/22 18:46

EAST Search History

S11	0	S6 S7 S8 S9 S10	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	WITH	ON	2006/05/22 18:45
S12	349	S6 S7 S8 S9 S10	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/22 18:45
S13	217	S6 S7 S8 S9 S10	US-PGPUB; USPAT	OR	ON	2006/05/22 18:46
S14	0	S12 not S13	US-PGPUB; USPAT	OR	ON	2006/05/22 18:45
S15	45	S13 and peptide	US-PGPUB; USPAT	OR	ON	2006/05/22 18:46
S16	3	montana-john-p$.inv.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	WITH	ON	2006/05/22 18:46
S17	10	coleman-patrick-f$.inv.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	WITH	ON	2006/05/22 18:46
S18	8	cole-carol-ann$.inv.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	WITH	ON	2006/05/22 18:46
S19	0	S6 S9 S16 S17 S18	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	WITH	ON	2006/05/22 18:47
S20	161	S6 S9 S16 S17 S18	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/22 18:47
S21	111	S6 S9 S16 S17 S18	US-PGPUB; USPAT	OR	ON	2006/05/22 18:48

EAST Search History

S22	20	blake-james.inv.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	WITH	ON	2006/05/22 18:48
S23	26	S22 S9 S16 S17 S18	US-PGPUB; USPAT	OR	ON	2006/05/22 18:49
S24	1	("5221736").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2006/05/22 21:30
S25	1	("5166050").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2006/05/22 21:30
S26	1	("4629783").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2006/05/23 13:17
S27	1	("20030215788").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2006/05/23 13:37
S28	2	wo-9015071$.did.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/23 14:15
S29	2	wo-8706005$.did.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/23 18:47
S30	2	("3798203").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2006/05/23 13:50
S31	2	JP-2002345467$.did.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/23 14:26
S32	0	ethylcarbamoyl with cystein with peptide	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/23 14:27

EAST Search History

S33	3	ethylcarbamoyl with cysteine with peptide	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/23 14:27
S34	20	blake-james.inv.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	WITH	ON	2006/05/23 15:39
S35	12	monji-nobuo$.inv.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	WITH	ON	2006/05/23 15:39
S36	3	montana-john-p$.inv.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	WITH	ON	2006/05/23 15:39
S37	10	coleman-patrick-f$.inv.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	WITH	ON	2006/05/23 15:39
S38	8	cole-carol-ann$.inv.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	WITH	ON	2006/05/23 15:39
S39	26	S34 S35 S36 S37 S38	US-PGPUB; USPAT	OR	ON	2006/05/23 15:39
S40	0	S39 and ehtylcarbamoyl	US-PGPUB; USPAT	OR	ON	2006/05/23 15:40
S41	3	S39 and ethylcarbamoyl	US-PGPUB; USPAT	OR	ON	2006/05/23 15:40
S42	89	cysteine with ethylcarbamoyl	US-PGPUB; USPAT	OR	ON	2006/05/23 15:40
S43	90	cysteine with ethylcarbamoyl	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/23 15:49

EAST Search History

S44	2	ethylcarbamoyl same immobil$ same $2protect$5	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/23 15:50
S45	1	ethylcarbamoy near25 $2protect$5	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/23 15:50
S46	147	ethylcarbamoyl near25 $2protect$5	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/23 15:54
S47	16590	peptide adj25 $2protect$5	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/23 15:54
S48	78	peptide adj25 immobili$ adj25 $2protect$5	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/23 16:14
S49	2	S48 and ethylcarbamoyl	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/23 15:54
S50	179	peptide with immobili$ with $2protect$5	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/23 19:06
S51	0	S49 not S48	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/23 17:08
S52	1	("4629783").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2006/05/23 17:57

EAST Search History

S53	1	"6322964"	US-PGPUB; USPAT	OR	ON	2006/05/23 18:08
S54	1	("4238620").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2006/05/23 18:24
S55	1	("4691006").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2006/05/23 18:26
S56	14	"peptide 39" and $ethylcarbamoyl	US-PGPUB; USPAT	OR	ON	2006/05/23 18:26
S57	19	"peptide 39" and $7ethylcarbamoyl$	US-PGPUB; USPAT	OR	ON	2006/05/23 18:28
S58	19	"peptide 39" and $7ethylcarbamoyl$	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/23 18:28
S59	1	("4111924").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2006/05/23 18:53
S60	2448212	s-(n-ethylcarbamoyl)	US-PGPUB; USPAT	OR	ON	2006/05/23 18:53
S61	0	"s-(n-ethylcarbamoyl)"	US-PGPUB; USPAT	OR	ON	2006/05/23 18:54
S62	1998	"(n-ethylcarbamoyl)"	US-PGPUB; USPAT	OR	ON	2006/05/23 18:54
S63	5406	ethylcarbamoyl	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/23 19:06
S64	3993	ethylcarbamoyl	USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/23 19:07
S65	67	ethylcarbamoyl with cysteine	USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/23 19:07

EAST Search History

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
		"s-(N-ethylcarbamoyl)	US-PGPUB; USPAT	OR	ON	2006/05/23 19:37
L1	101	Blake-James$.inv.	US-PGPUB; USPAT	OR	ON	2006/05/25 21:23
L2	4	cole-Carol-A$.inv.	US-PGPUB; USPAT	OR	ON	2006/05/25 21:22
L3	5	Coleman-Patrick-F$.inv.	US-PGPUB; USPAT	OR	ON	2006/05/25 21:22
L4	9	Monji-Nobuo$.inv.	US-PGPUB; USPAT	OR	ON	2006/05/25 21:22
L5	2	Montana-John-P$.inv.	US-PGPUB; USPAT	OR	ON	2006/05/25 21:22
L6	16	Blake-James.inv.	US-PGPUB; USPAT	OR	ON	2006/05/25 21:23
L7	7	Blake-James-F.inv.	US-PGPUB; USPAT	OR	ON	2006/05/25 21:23
L8	33	2 3 4 5 6 7	US-PGPUB; USPAT	OR	ON	2006/05/25 21:23
L10	1	("20030215788").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2006/05/25 21:46
S1	0	"s-(N-ethylcarbamoyl)"	US-PGPUB; USPAT	OR	ON	2006/05/23 19:38
S2	13142	$ethylcarbamoyl	US-PGPUB; USPAT	OR	ON	2006/05/23 19:38
S3	14781	$ethylcarbamoyl	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/23 19:39
S4	6	$ethylcarbamoyl near cysteine	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/23 19:51
S5	1	("5075211").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2006/05/23 19:54
S6	2	"6485900"	US-PGPUB; USPAT	OR	ON	2006/05/23 20:20

EAST Search History

S7	1	("6214539").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2006/05/23 19:55
S8	1	("20030211117").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2006/05/23 19:55
S9	5	coleman-patrick-F$.inv.	US-PGPUB; USPAT	OR	ON	2006/05/23 20:24
S10	45	coleman-patrick-$.inv.	US-PGPUB; USPAT	OR	ON	2006/05/23 20:25
S11	45	coleman-patrick$.inv.	US-PGPUB; USPAT	OR	ON	2006/05/23 20:26
S12	40	S11 not S9	US-PGPUB; USPAT	OR	ON	2006/05/23 20:25
S13	0	coleman-patrick.inv.	US-PGPUB; USPAT	OR	ON	2006/05/23 20:26
S14	1	coleman-patrick.inv.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	ON	2006/05/23 20:27
S15	72	coleman-patrick$.inv.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	ON	2006/05/23 20:27
S16	10	coleman-patrick-F$.inv.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	ON	2006/05/23 20:27
S17	5	S16 not S9	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	ON	2006/05/23 20:30
S18	0	"SYNTHETIC ANTIGEN FOR THE DETECTION OF ANTIBODIES".ti.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	ON	2006/05/25 20:11
S19	52	"SYNTHETIC ANTIGEN".ti.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	ON	2006/05/23 20:38

EAST Search History

S20	0	S19 and ethylcarbamoyl	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	ON	2006/05/23 20:39
S21	21	S19 and cysteine	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	ON	2006/05/23 20:42
S22	4	"thiol-protected peptides"	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	ON	2006/05/23 20:44
S23	1	("4111924").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2006/05/23 20:44
S24	2	("3798203").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2006/05/23 21:00
S25	3063	"protected peptide"	US-PGPUB; USPAT	OR	ON	2006/05/23 21:00
S26	3452	"protected peptide"	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/23 21:00
S27	1	"protected peptide immobilized"	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/23 21:05
S28	13	"protected peptide" with immobil$5	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/23 21:05
S29	13	"protected peptide" with immobil$7	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/23 21:10

EAST Search History

S30	393	$2protect$5 near immobiliz$7	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/23 21:10
S31	389	S30 not S29	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/23 21:11
S32	32	S31 and cysteine	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/23 21:24
S33	9	ethylcarbomyl	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/23 21:24
S34	5	(US-5075211-$ or US-4629783-$ or US-6130314-$ or US-6485900-$). did. or (US-3798203-$).did.	USPAT; USOCR	OR	ON	2006/05/23 21:24
S35	0	S34 and S33	USPAT; USOCR	OR	ON	2006/05/23 21:24
S36	5406	ethylcarbamoyl	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/23 21:26
S37	4	S34 and "39"	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/23 21:25
S38	1	S34 and S36	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/23 21:25

EAST Search History

S39	16127	$7ethyl$1carbamoyl$5	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/23 21:40
S40	10477	$7ethyl$1carbamoyl$5	USPAT	OR	ON	2006/05/23 21:28
S41	1	S4 and peptide	USPAT	OR	ON	2006/05/23 21:29
S42	2	S4 and protein	USPAT	OR	ON	2006/05/23 21:28
S43	1995	S40 and peptide	USPAT	OR	ON	2006/05/23 21:29
S44	554	S43 and cysteine	USPAT	OR	ON	2006/05/23 21:40
S45	0	S-ethylcarbamoyl-cysteine	USPAT	OR	ON	2006/05/23 21:40
S46	1	S-ethylcarbamoyl-cysteine	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/23 21:40
S47	2	ethylcarbamoyl-cysteine	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/23 21:40
S48	4	ethylcarbamoyl$cysteine	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/23 21:42
S49	13	$ethylcarbamoyl$cysteine not $methylcarbamoyl$cysteine	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2006/05/23 21:42
S50	2	wo-8706005$.did.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	ON	2006/05/25 21:22

NPL___ ADONIS_1/_ MIC___ BioT_1/_Main__NO__Vol NO__ NOS___CKCite__Dup__Int WK

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1.Mani et al. Effect of HIV-1 peptide presentation on the affinity constants of two monoclonal antibodies determined by BIAcore technology. Mol Immunol. 1994 Apr;31(6):439-44.

2. Blake et al., "Ethylcarbamoyl Protection for Cysteine in the Preparation of Peptide-Conjugate Immunogens", Int. J. Pept. Protein Res. 40:62-65 (1992).

Thank you.

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Int. J. Peptide Protein Res. 40, 1992, 62–65

Ethylcarbamoyl protection for cysteine in the preparation of peptide-conjugate immunogens

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During the solid-phase synthesis of over 100 peptides, we have observed that the ethylcarbamoyl group is useful for the side chain protection of cysteine in peptides containing a single cysteine residue. The ethylcarbamoyl group is stable to the conditions of acidolytic cleavage, purification and long term storage. Brief treatment of peptides containing an S-ethylcarbamoylcysteine residue with aqueous sodium hydroxide gives the deprotected cysteine peptide that can be coupled to carrier molecules such as proteins to give immunogen conjugates.

Key words: cysteine; ethylcarbamoyl; immunogen; peptide-protein conjugate; solid-phase peptide synthesis

A common application of synthetic peptides has been their specific conjugation to carrier molecules, such as proteins, to obtain immunogens that can be used to raise antibodies against the peptides (1, 2). The preferred conjugation site is the thiol function of cysteine because of its rapid and specific reaction with proteins modified to contain maleimide groups (3). The side chain thiol of cysteine is distinct among the amino acid side chains because of its instability, principally due to ease of oxidation. This tendency is most pronounced at neutral or alkaline pH, but can still be a long term problem at acidic conditions, e.g., storage of peptides that have been lyophilized from acid solution. For the preparation of immunogens conjugated through cysteine, it is desirable to be able to synthesize peptides with the cysteine thiol protected by a group that is stable to the conditions of cleavage, deprotection, purification and storage, but which can easily be removed to give the free thiol group.

Our experience with the ethylcarbamoyl (Ec) group indicates that it is useful for this purpose. Although it has been twenty-five years since the Ec group was first proposed for the protection of cysteine by Guttman (4), it has been infrequently used in standard solid-phase synthesis of peptides (5, 6) or in alternative solid-phase strategies (7). We now report our results on the solid-phase synthesis (8) of peptides containing a single cysteine residue protected with the Ec group and the subsequent use of these peptides for the preparation of peptide-conjugate immunogens.

MATERIALS AND METHODS

Solid-phase peptide synthesis was performed on an Applied Biosystems Peptide Synthesizer Model 430A using Boc amino acids. Typically 0.35–0.40 mmol of MBHA resin was used and 2 mmol of Boc-amino acid was coupled as recommended by ABI – either DCC/HOBt or as the preformed symmetric anhydride. Side chain protection was as follows: Ser, Bzl; Thr, Bzl; Glu, Bzl; Asp, Bzl or cyclohexyl; Tyr, 2-BrZ; Lys, 2-ClZ; Arg, Tos; His, Z or Bom; Met, sulfoxide or unprotected; Trp, formyl; Cys, Ec. Boc-Cys(Ec)-OH was coupled to the resin by reaction with dicyclohexylcarbodiimide/1-hydroxybenzotriazole by the same cycles used for Boc-Asn – either a single couple at the carboxyl terminus or a double couple at the amino terminus.

For cleavage and deprotection the peptide resin was treated with 90% HF/10% anisole (9) at 0° for 1 h or by the low-high method (10) with or without *p*-thiocresol, depending on whether formyltryptophan was or was not present. After evaporation of HF the peptide-resin mixture was washed with ethyl acetate and the peptide was dissolved in 50% acetic acid. For acidic peptides that had low solubility in acid the peptide was dissolved in 30 mM ammonium bicarbonate with additional 1N

Abbreviations: Boc, *tert.*-butyloxycarbonyl; Ec, ethylcarbamoyl.

ammonium hydroxide being added to give pH 7–8. At this step it was important to carefully monitor pH to assure that too high a pH did not give unwanted deprotection of the Cys(Ec) group. Partial purification of the crude peptides was obtained by gel filtration on Sephadex G-25 in 20% acetic acid or 30 mM ammonium bicarbonate.

Reversed-phase HPLC was performed on a Waters Model 600E instrument. Analytical runs were done on a Brownlee Aquapore RP-300 column, 4.6 × 250 mm. Preparative runs were done on a Rainin Dynamax-300A, C-8 column, 22 × 250 mm. Elution of the samples was by a gradient of acetonitrile in 0.1% trifluoroacetic acid, and peptide was detected by monitoring the absorbance at 214 nm. The peptides were lyophilized and stored at 4° until they were conjugated.

Deprotection of Cys(Ec) peptides

Although the great majority of Cys(Ec) peptides could be deprotected by our standard conditions, there were three or four peptides that required a higher concentration of sodium hydroxide. Consequently, prior to a conjugation experiment a small sample (ca. 0.5 mg) of peptide was dissolved in 50 μl of water and 10 μl of 1N sodium hydroxide were added. Twenty-microliter aliquots were removed after 1 and 3 min and immediately added to 5 μl of 1M monobasic potassium phosphate. Analysis for thiol S—H by the Ellman method (11) indicated typically 60–70% of the theoretical amount on the basis of peptide weight. Since peptide content of lyophilized powders is usually 60–70% and the thiol values were essentially the same after 1 and 3 min, this indicated that deprotection of the Ec group was nearly complete. In those few cases where the preliminary experiment indicated incomplete deprotection the experiment was repeated using a higher concentration of sodium hydroxide. For scale-up of the deprotection and the bimolecular coupling reactions the volume of the deprotection solution was kept as low as possible. Therefore although the volume of 1N sodium hydroxide used was proportional to the weight of the peptide in the small-scale experiment, the concentration of sodium hydroxide was increased to 0.5 N.

N-tert.-butyloxycarbonyl-S-ethylcarbamoyl-L-cysteine. This is a modification of an unpublished synthesis of D. Yamashiro, which was itself a modification of published syntheses (4, 12).

Cysteine-free base (36.1 g, 0.298 mol) was suspended in 300 mL dimethylformamide. The mixture was stirred at 0° and 22.9 mL trifluoroacetic acid were slowly (10 min) added, followed by a slow addition of 26.7 mL ethyl isocyanate. The mixture was stirred at 0° for 15 min and at room temperature for 16 h. The dimethylformamide was evaporated *in vacuo* and the residue was dissolved in 270 mL of cold water. The aqueous solution was washed with two 150-mL portions of ether, cooled to 0°, and 5 N sodium hydroxide was added to give pH 7.0. Absolute ethanol (800 mL) was added and the mixture was stored overnight at 4°. Filtration, followed by washing with cold ethanol and ether, and brief drying, gave 28.8 g of a white solid.

The above solid was suspended in 300 mL dimethyl sulfoxide and 25.5 mL *N,N*-diisopropylethylamine was added, followed by the slow addition (15–20 min) of 33.9 g of solid di-*tert.*-butyl dicarbonate. The reaction mixture was stirred for 2 h at room temperature. Then 600 mL of ice/water and 150 mL of saturated sodium chloride were added. After stirring at 0° for a few minutes 3 N hydrochloric acid was added to give pH 2–3. The aqueous mixture was extracted with 350 mL ethyl acetate. The pH of the aqueous fraction was readjusted to less than 3 and then extracted again with 300 mL ethyl acetate. The combined organic extracts were washed with 40 mL of saturated sodium chloride, dried over magnesium sulfate, and evaporated to a residue that crystallized. Ethyl acetate (45 mL) and 230 mL *n*-hexane were added and the mixture was stored at 4° overnight. The solid was filtered, washed with *n*-hexane, and dried briefly *in vacuo* to give 60 g of product. The product was dissolved in 350 mL of boiling 20% ethanol/water. Then 45 mL water was added and the solution was allowed to cool. After storage at 4° for several hours the mixture was filtered and the solid product was washed with cold 20% ethanol/water, and dried. The yield was 31.29 g (0.107 mol). The melting point was 143–145°; reported 139–140° (12) and 139° (13). The optical rotation was $[\alpha]_D$ −21.2° (*c* 1, ethanol); reported −21.8° (12) and −22.2° (13).

Thin-layer chromatography on silica gel in chloroform:acetic acid (15:1) gave a single chlorine positive spot at R_f 0.2.

Anal. calc. for $C_{11}H_{20}N_2O_5S$: C 45.19, H 6.90, N 9.58, S 10.96. Found: C 45.13, H 6.84, N 9.52, S 10.73.

RESULTS AND DISCUSSION

Peptide synthesis

Boc-Cys(Ec)-OH was available by synthesis (see Materials and Methods) and could be readily coupled to methylbenzhydrylamine (MBHA) resin by reaction with dicyclohexylcarbodiimide/1-hydroxybenzotriazole. In cases where an additional charge at the carboxyl terminus was desired, Boc-PAM-Gly resin could be substituted for MBHA resin. Thereafter the peptide chain was assembled by the standard conditions of the Boc strategy of solid-phase peptide synthesis (8).

Peptides were cleaved from the resin and their side chains were deprotected, with the exception of cysteine, by reaction with hydrogen fluoride/anisole (9) or by the low-high hydrogen fluoride method (10). Crude peptides were partially purified by gel filtration in aqueous acetic acid, or in ammonium bicarbonate in the unusual cases where the peptides had low solubility in acid. In the latter case a few percent deprotection of the Ec group could be detected. For final purification reversed-

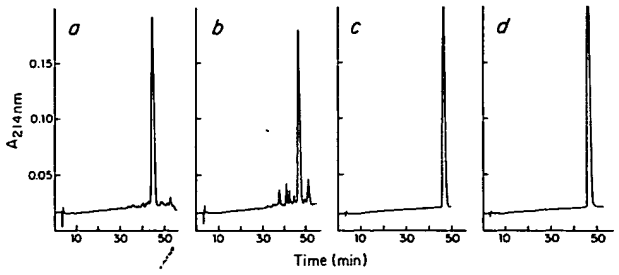

FIGURE 1

Reversed-phase HPLC of: (a) crude peptide I; (b) crude peptide Ic; (c) HPLC purified Ic; (d) HPLC purified Ic that had been stored at 4° for 11 months. The gradient was 10–50% acetonitrile in 0.1% trifluoroacetic acid over 50 min.

phase HPLC in the trifluoroacetic acid-acetonitrile system was preferred. Molecular weight determination by plasma desorption mass spectroscopy gave the correct value for the peptide with the Ec group on the cysteine for all preparations that were tested. Approximately 120 peptides containing a Cys(Ec) residue have been routinely synthesized.

In the three peptide sequences that were assembled with or without a carboxyl terminal Cys(Ec) residue we observed that unreacted amine after coupling, as determined by the ninhydrin test (14), was slightly (0.5–1%) higher on average throughout the assembly for the sequences that contained the Cys(Ec) residue. It is unclear whether this effect was an artifact of the ninhydrin reaction, represented a partial decomposition of the Ec group, or was an effect on peptide conformation that reduced the accessibility of the terminal amino group to coupling reagent. Analysis by reversed-phase HPLC for the peptide pair Leu-Lys-Lys-Leu-Leu-Lys-Leu-Leu-Lys-Lys-Leu-Leu-Lys-Leu (I) (15) and Leu-Lys-Lys-Leu-Leu-Lys-Leu-Leu-Lys-Lys-Leu-Leu-Lys-Leu-Cys(Ec) amide (Ic) is shown in Fig. 1. The results indicate that: 1) there were more impurities present in crude Ic than in I; 2) subsequent purification of Ic gave a product whose retention time in analytical HPLC was unchanged; 3) Ic was stable to long-term storage.

When it was desired to conjugate the peptide at its amino terminus the Cys(Ec) residue could be incorporated at the amino end of the peptide chain, usually followed by an acetylation step. In these cases unreacted amine after coupling of Boc-Cys(Ec)-OH, as measured by the ninhydrin test, showed a sudden significant (ca. 5%) increase. Analysis by HPLC of the peptides Leu-His-Gly-Pro-Glu-Ile-Leu-Asp-Val-Pro-Ser-Thr (II) and Gly-Cys(Ec)-Gly-Gly-Gly-Leu-His-Gly-Pro-Glu-Ile-Leu-Asp-Val-Pro-Ser-Thr (IIc) showed only minor differences in heterogeneity between the crude peptides and indicated that IIc was stable to purification and storage (Fig. 2). In this connection it should be mentioned that although the reactivity to base of the Cys(Ec) residue precludes its use at the carboxyl terminus of a peptide that is assembled by the Fmoc strategy, it is possible to couple Boc-Cys(Ec)-OH to the amino terminus of the assembled peptide and obtain the amino terminal Cys(Ec) peptide after standard Tfa cleavage and deprotection. We have done this a few times and the product was easily purified and stable to storage.

It should be parenthetically noted that incorporation of several Cys(Ec) residues in a peptidyl resin gave poor coupling yields *throughout the assembly*, as determined by ninhydrin and that this was reflected in the heterogeneity of the cleaved product. It is evident that there is a minor problem with the use of the Cys(Ec) residue – an effect on peptide conformation or a partial decomposition – that is compounded by the use of several Cys(Ec) residues in the middle of the peptide chain. Therefore the usefulness of Ec protection for the preparation of peptides containing several Cys-Cys disulfide bridges is questionable.

Deprotection of Cys(Ec) residues

The Ec group can be removed by reaction with alkaline nucleophiles such as hydroxide ion, ammonia and hydrazine (4). The first of these was the most convenient. Typically 0.15–0.5 N sodium hydroxide was used to

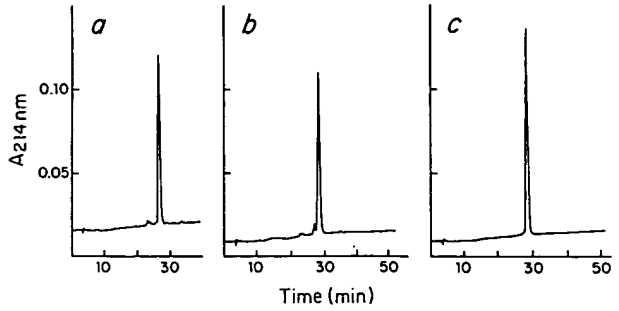

FIGURE 2

Reversed-phase HPLC of: (a) crude peptide II; (b) crude peptide IIc: (c) HPLC purified IIc that had been stored at 4° for 11 months.

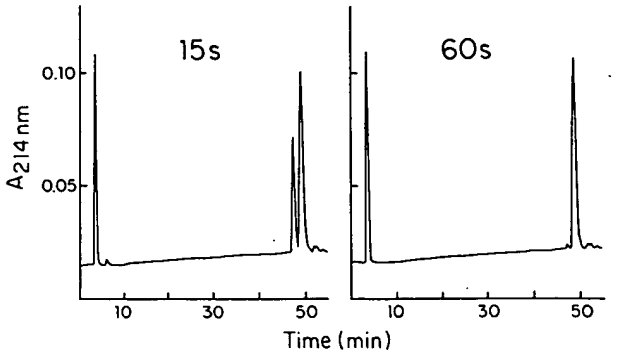

FIGURE 3

Reversed-phase HPLC of a sample of Ic that was treated with 0.17 N sodium hydroxide for 15 or 60 s.

deprotect the cysteine residue. HPLC analysis (Fig. 3) of the deblocking of peptide Ic indicated greater than 95% completion in one minute although 2–3 min deblocking were routinely used for most peptides. For peptides that contained asparagine in base-sensitive sequences such as Asn-Gly or Asn-Ala, the time for deprotection was reduced to one minute.

After the prescribed deprotection time the peptide was immediately conjugated to a freshly prepared sample of a protein that had been reacted with sulfosuccinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate and the resulting peptide-protein conjugate was isolated after gel filtration or dialysis.

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EFFECT OF HIV-1 PEPTIDE PRESENTATION ON THE AFFINITY CONSTANTS OF TWO MONOCLONAL ANTIBODIES DETERMINED BY BIAcore™ TECHNOLOGY

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Abstract—We studied two monoclonal antibodies (MAbs 9-11 and 41-1) which are specific for dominant and conserved epitopes located on HIV-1 transmembrane Gp41. These MAbs recognize both Gp41 and a synthetic HIV-1 envelope peptide (39GC) which is a fragment of Gp41. The interactions between MAbs 9-11 and 41-1 and 39GC either coupled to a sensor chip or to alkaline phosphatase were investigated using BIAcore™ technology. The association and dissociation rate constants as well as the affinity constants were determined. BIAcore™ technology allows real-time determination of the interaction between two molecules without the need for any labeling, neither isotopic nor enzymatic. The peptide 39GC was immobilized by coupling to dextran on the BIAcore™ biosensor through a disulfide bond with a cysteine residue added to the N-terminus of the synthetic peptide. The two native cysteine residues located in the loop of Gp41 were protected by ethylcarbamoyl residues ($CONHC_2H_5$); this chemical modification prevented the formation of the S–S bridge and in particular the internal loop. We specifically studied the interaction between the MAbs and either the protected peptide or the peptide whose cysteine residues had been deprotected *in situ* by alkaline treatment. The results showed that MAb 41-1 recognized 39GC either protected ($Ka = 7.6 \times 10^6\ M^{-1}$) or unprotected ($Ka = 1.48 \times 10^8\ M^{-1}$), whereas MAb 9-11 recognized only the unprotected form ($Ka = 2.18 \times 10^8\ M^{-1}$). Our results suggest that the epitope MAb 9-11 is directed against a part of the peptide sequence which includes the two native cysteines. The difference in affinity observed for MAb 41-1 between the protected and the unprotected forms of 39GC was found to be due to a lower rate of dissociation for unprotected 39GC; these results illustrate the importance of peptide conformation on antibody recognition and might be explained by a conformational change due to reconstitution of the internal loop following deprotection of the thiol groups. MAbs 9-11 and 41-1 also recognized 39GC conjugated to alkaline phosphatase and deprotected. We observed a difference between the rate constants for MAb 41-1 binding to free peptide and its binding to the peptide–enzyme conjugate which might be due to changes in peptide flexibility. In contrast, the rate constants of MAb 9-11 were the same in both experiments, suggesting that the rigidity of the internal loop prevents changes in 9-11 epitope conformation.

Key words: HIV-1, BIAcore™, peptide, immunoassay, monoclonal antibodies, conjugate, epitope analysis, kinetic rate constants.

INTRODUCTION

One of the most immunodominant epitopes of HIV-1 is present in a conserved region of the virus located on the Gp41 transmembrane protein (aa 598–609) (Gnann *et al.*, 1987; Wang *et al.*, 1986; Zvelebil *et al.*, 1988). The tertiary structure of Gp41 has been reported (Gallaher *et al.*, 1989) and the importance of a native cystine located in this sequence, a loop formed by C_{603} and C_{609}, has been underscored (Gallaher *et al.*, 1989; Lacroix *et al.*, 1989).

The diagnosis of HIV infection is a challenge, particularly with respect to the development of reliable and sensitive means for early detection of anti-HIV antibodies. For that purpose, the use of peptides mimicking this Gp41 fragment appears to be a strategy of choice (Fenouillet *et al.*, 1990). An assay format using a synthetic HIV-1 envelope peptide (39GC) coupled to alkaline phosphatase has been studied to develop an immunoassay for anti-Gp41 on the new ACCESS® instrument (De Kerdaniel *et al.*, 1993). The phenomenon of conformational changes of proteins due to adsorption or conjugation is well established (Abraham *et al.*, 1991; Shields *et al.*, 1991). This phenomenon is even more important for small antigenic peptides.

We studied herein the interaction of two monoclonal antibodies (MAbs 41-1 and 9-11) raised against Gp160, precursor of Gp41, with the HIV-1 peptide linked to a solid-phase or in solution, coupled to alkaline phosphatase. Together with the conformational change induced by the support, we studied the influence of the internal loop of the peptide on antibody recognition.

The real-time biospecific interaction analysis made possible by a new biosensor technology (BIAcore™),

using surface plasmon resonance (Dubs *et al.*, 1991; Jonsson *et al.*, 1991; Malmquist, 1993), allowed simple and rapid direct monitoring of anti-HIV MAb–peptide interactions. Association and dissociation kinetic constants were also calculated.

MATERIALS AND METHODS

Monoclonal antibodies (MAbs)

MAbs, raised against Gp160, precursor of Gp41, were produced in mouse ascites and partially purified by ammonium sulfate precipitation. MAb 9-11 (IgG_1) was obtained from Dr J.-C. Mazié (Institut Pasteur, Paris, France) and MAb 41-1 (IgG_1) from Dr K. Shriver (Genetic Systems Corp., Seattle, U.S.A.).

Synthetic peptide (39GC)

Peptide fragment aa 584–609 [CGGRILAVERYL-KDQQLLGIWGC(SEC)SGKLIC(SEC)] was derived from the HIV-1 BRU isolate Gp41 protein. A CGG amino acid sequence was purposely added to the N-terminal end of the peptide to provide a thiol moiety directly available for solid-phase or alkaline phosphatase coupling. S-ethylcarbamoyl (SEC) cysteines were used in place of the two native internal cysteines. The use of the S-ethylcarbamoyl group, which can be easily cleaved (Storey *et al.*, 1972) by mild alkaline treatment (NaOH, pH 9.7), allows modification of the native loop and secondary structure of the peptide. The use of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), a sensitive sulfhydryl reagent, allowed us to control the oxidation state of the cysteine residues (Anderson and Wetlaufer, 1975). DTNB is cleaved by free thiol, leading to the liberation of 2-nitrobenzoic acid; this reaction can be easily quantified by measuring the absorption at 412 nm ($\varepsilon = 14150\ M^{-1}\,cm^{-1}$) after adding 50 μM DTNB to a fresh solution of peptide.

The peptide (39GC) was synthesized by the automated Merrifield solid-phase technique using an Applied Biosystems 430A peptide synthesizer as described by Barany and Merrifield (1980). After cleavage by HF, the crude peptide extracted by 50% acetic acid was lyophilized. Purification to >90% was performed by reversed-phase HPLC; the peptide was eluted with a gradient of acetonitrile–water–0.1% trifluoroacetic acid.

Peptide–alkaline phosphatase conjugate

Alkaline phosphatase (4 mg, 10 mg/ml) from Boehringer Mannheim was activated with a 30-fold excess of sulfosuccinimidyl 4-(*N*-maleimolarmidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC, Pierce), for 30 min at 20°C. The mixture was purified to remove the excess of linker by gel filtration chromatography using Sephadex G25 prepacked PD-10 columns (Pharmacia) equilibrated with 5 mM borate buffer, pH 6.5, containing 2 mM $MgCl_2$. Incorporation of maleimide was determined using a known amount of 2-mercaptoethanol, then 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) for the assay of excess thiol; this procedure revealed 1.5–3 reactive maleimide groups per enzyme molecule.

Peptide 39GC (1 mg) was dissolved in 0.1 ml of deaerated H_2O just before use and added under gentle stirring to a 3 mg activated alkaline phosphatase deaerated solution. The coupling reaction was conducted at 20°C for 2.5 hr under an argon atmosphere. The S-ethylcarbamoyl groups were cleaved by mild alkaline treatment (pH 9) for 1 hr (Storey *et al.*, 1972). The coupling mixture was purified by FPLC (gel exclusion chromatography) using a Superose 6 column (Pharmacia). The collected fractions were assayed for enzymatic activity using *p*-nitrophenylphosphate substrate. The most active fractions containing the peptide–enzyme conjugate were pooled.

BIAcore™ technology

The biosensor BIAcore™ system allows real-time interaction analysis without the need for any labeling, neither isotopic nor enzymatic. Biosensor technology is based on optical surface plasmon resonance (SPR) for detecting small changes in the refractive index on the surface of a thin gold film coated with a dextran matrix to which one of the reactants is covalently linked, while the other one is introduced in a flow passing over the surface. The refractive index (R_A) resulting from the interaction of the reactant molecule is expressed in resonance units (RU): an SPR response of 1000 RU corresponds to a surface concentration of 1 ng of reactant per mm^2 of the 100 nm thick dextran layer.

The running and dilution buffer in all experiments was 10 mM Hepes-buffered saline (pH 7.4) with 3.4 mM EDTA and 0.005% BIAcore™ surfactant. Regeneration of the sensor chip was performed using 15 μl of 100 mM HCl.

Peptide 39GC was immobilized on a sensor chip CM5 (Jonsson *et al.*, 1991) through an S–S bond. The sensor chip surface was activated with EDC/NHS [100 mM *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride/400 mM *N*-hydroxy succinimide]; 80 mM PDEA [2-(2-pyridinyldithio)ethaneamine] was added in borate buffer (pH 8.6) and ethanolamine was injected for the deactivation of the remaining activated groups. Peptide 39GC (50 μg/ml) was injected in acetate buffer (pH 6.0), then 50 mM cysteine in 1 M NaCl was added for deactivation of the remaining PDEA groups. The binding kinetics of the MAbs were determined by injecting increasing concentrations of each MAb at a flow rate of 2 μl/min; association and dissociation of the MAb was visualized by the sensorgram. Dissociation was observed in running buffer, without dissociating agents. Peptide deprotection was achieved *in situ* by injecting 20 μl NaOH (pH 9.7).

Rabbit anti-mouse Fc (RAM-G1, Pharmacia), 25 mg/ml in acetate buffer (pH 5.0), was immobilized by coupling to EDC/NHS activated dextran. MAbs 9-11 or 41-1 were bound to RAM-G1 and the binding of peptide 39GC-alkaline phosphatase conjugate to each MAb was monitored. MAb–conjugate interactions were determined in channels with high concentration coat

(10 ng/mm^2), while binding kinetics were studied in channels with low concentration coat (4 ng/mm^2).

Epitope mapping (Fagerstam *et al.*, 1990) was performed by sequentially adding the MAbs to the immobilized peptide, deprotected or not by NaOH treatment. In the case of the peptide–alkaline phosphatase conjugate, epitope mapping was performed pairwise on immobilized RAM-G1.

Kinetic runs were performed using six different ligand concentrations. The amount of antibody bound (R_A) to the peptide as well as the reaction rate (dR_A/dt) are given by the Bialogue™ software. Kinetic rate constants (k_{on} and k_{off}) as well as apparent equilibrium affinity constants ($K_A = k_{on}/k_{off}$) were determined using Bialogue™ Kinetics Evaluation software.

RESULTS

Control of peptide loop conformation

Peptide 39GC was synthesized by using S-ethylcarbamoyl protected internal cysteines (see Materials and Methods). We determined the oxidation state of the cysteines with DTNB. After addition of 50 μM DTNB to a fresh solution of peptide (5 μM in H_2O), we found 4.4 ± 0.2 μM free N-terminal cysteines, which corresponds to 0.88 ± 0.4 free thiol per peptide. When sodium hydroxide was added to the protected peptide to a pH of 9–10 in the presence of DTNB, the A_{412} value increased rapidly to reach, after 10–20 min, a plateau corresponding to 15.5 ± 0.5 μM free cysteines (3.1 ± 0.1 thiol per peptide). This showed that the mild alkaline treatment cleaved the S-ethylcarbamoyl protective groups. Furthermore, the addition of DTNB to the protected peptide (5 μM) treated at pH 9–10 for 10 min showed only 9.5 ± 0.5 μM of reactive cysteines. This result suggests that 60% of the total cysteines were oxidized to give disulfide bonds. The native internal loop formation is probably favored but the possibility of peptide dimerization cannot be excluded. These experiments demonstrated that it is possible to easily control the cysteine state in peptide 39GC to study the influence of the conformation of this peptide on antibody recognition.

Peptide epitope study

MAb binding to peptide 39GC. The two MAbs were tested for their ability to bind to peptide 39GC. The amount of antibody binding was measured with the BIAcore system using a sensor chip coated with the peptide (3.5 ng/mm^2) through an S–S bond with the N-terminal cysteine added to the HIV peptide sequence. First, we investigated the binding of the MAbs to the peptide containing the two S-ethylcarbamoyl-protected cysteines as synthesized. MAb 41-1 (4 μl, 50 μg/ml) bound to the 39GC peptide gave a signal of 420 RU. Under the same conditions, MAb 9-11 (4 μl, 50 μg/ml) did not significantly bind to the peptide as shown by a signal of 31 RU which is in the range of the background value obtained with an unrelated MAb.

To study the influence of the cysteine thiol protection on MAb binding, we removed the S-ethylcarbamoyl groups directly on the dextran-linked peptide by mild alkaline treatment (NaOH pH 9.7, 2 × 10 min). Interestingly, the binding of MAb 41-1 was found to be only slightly increased (568 RU). In contrast, the recognition of MAb 9-11 appeared to be very sensitive to internal cysteine deprotection since a signal of 425 RU was obtained.

Epitope mapping. Information on the relative location of the two 39GC epitopes was obtained by sequential addition of MAbs 41-1 and 9-11 to the deprotected peptide. We showed that both MAbs were able to bind simultaneously to deprotected 39GC, indicating that the two MAbs bind to two independent epitopes. The epitope recognized by MAb 9-11 probably includes part of the peptide internal loop since no MAb 9-11 binding to the SH-protected peptide was observed.

Kinetic measurements. The interaction of MAbs 41-1 and 9-11 with peptide 39GC was further studied by measuring the association and dissociation rate constants. The antibody was allowed to interact with the peptide covalently linked to the sensor chip (1,2 ng/mm^2). The antibody at concentrations ranging from 15 to 1300 nM was injected at a constant flow rate (2 μl/min). Table 1 shows the kinetic rate constants (k_{on} and k_{off}) and the apparent equilibrium affinity constants ($K_A = k_{on}/k_{off}$) calculated from dR_A/dt versus R_A plots. Association rate constants were calculated from data point fitting to a line with a correlation coefficient superior to 0.9. Kinetic runs were performed for six or seven MAb concentrations.

Concerning MAb 41-1, it should be observed that deprotection of the peptide increased the equilibrium constant not because of association rate change but because of the dramatic decrease of the dissociation rate. Since binding measurements showed how sensitive the MAb 9-11 was to protection of the internal cysteine, it was only possible to determine the kinetic constants for its binding to the peptide treated with NaOH. Finally, we observed that the kinetic parameters of both MAbs vis-a-vis the deprotected peptide were very similar.

Peptide–enzyme conjugate binding

Epitope determination. We studied the capacity of the soluble peptide–enzyme conjugate to bind to MAbs 41-1 and 9-11 immobilized through their Fc region to rabbit anti-mouse Fcγ1 Ab (RAM-G1) covalently attached to the dextran matrix (10 ng/mm^2). The two MAbs bound tightly to the RAM-G1 surface giving a reproducible signal (Table 2) which was within the usual range for the technique.

The peptide–alkaline phosphatase conjugate (10 μl, 50 μg/ml) bound to both MAbs very similarly. Table 2 also shows that $36.3 \pm 2\%$ of immobilized MAb 41-1 captured one conjugate molecule, and $36.4 \pm 3\%$ of MAb 9-11 captured one conjugate molecule. This demonstrated that the two MAb epitopes were accessible in the conjugated peptide as in the deprotected peptide.

Table 1. Kinetic parameters for the binding of MAbs 41-1 and 9-11 to the peptide 39GC immobilized on the solid phase or coupled to alkaline phosphatase in solution

Analyte[a]	Immobilization[b]	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_A (M^{-1})
MAb 41-1	Peptide 39GC	17.9×10^3	234×10^{-5}	0.76×10^7
MAb 41-1	Peptide 39GC deprotected[c]	7.45×10^3	5.02×10^{-5}	14.8×10^7
MAb 9-11	Peptide 39GC	ND[d]	ND	ND
MAb 9-11	Peptide 39GC deprotected[c]	23.1×10^3	10.6×10^{-5}	21.8×10^7
Conjugate	MAb 41-1	21.2×10^3	68.6×10^{-5}	3.09×10^7
Conjugate	MAb 9-11	29.9×10^3	14.8×10^{-5}	20.8×10^7

[a]The analyte was injected at a flow rate of 2 µl/min at concentrations ranging from 15 to 1300 nM.

[b]The peptides were immobilized via the thiol moiety of the N-terminal cysteine residue (see Materials and Methods section); the MAbs were bound to a rabbit anti-mouse Fc Ab immobilized on the dextran surface (RAM-G1 surface).

[c]Internal peptide cysteines were deprotected (S-ethylcarbamoyl blocking group cleavage) by a 10 min NaOH pH 9.7 treatment of the peptide-linked surface.

[d]Not determined because the binding was too low.

Interestingly, when the same MAb was injected a second time after binding of the conjugate to the immobilized antibody, almost no RU signal was observed (Table 2), suggesting that only one peptide residue per conjugate molecule was accessible (mean value: 1.15 ± 0.05). When the second injected MAb was different from the immobilized one, a significant RU signal was observed (Table 2), showing that the enzyme-linked peptide contained the two independent MAb epitopes, like the free deprotected peptide.

Kinetic measurements. The association and dissociation rate constants were measured by injecting different concentrations of conjugate on MAb 41-1 or 9-11 immobilized onto RAM-G1 (4 ng/mm²). Table 1 shows the calculated rate and apparent equilibrium constants. The rate constants of the conjugate binding to MAb 41-1 are different from those obtained for the MAb binding to untreated and alkaline pH-treated peptide: the equilibrium affinity constant was intermediate between those obtained for the MAb binding to untreated and alkaline pH-treated peptide. MAb 9-11 showed rate constants and therefore equilibrium affinity constants for conjugate binding very close to those obtained with alkaline pH-treated peptide.

Table 2. Binding of MAbs 41-1 and 9-11 to the enzyme-linked peptide conjugate using a rabbit anti mouse Fc Ab attached to the BIAcore™ surface

Injection 1[a] MAb (RU)	Injection 2 Blocking MAb[b] (RU)	Injection 3 Conjugate (RU/S_1[c])	Injection 4 MAb (RU/S_2[d])
41-1/951	95	356/0.374	41-1/67/0.188
9-11/1232	39	483/0.392	9-11/59/0.122
41-1/813	85	295/0.363	9-11/223/0.756
9-11/1197	39	405/0.338	41-1/126/0.311

[a]The sequential injections of the different analytes were performed as follows: 10 µl of antibodies and conjugate (50 µg/ml) 10 µl were injected in 5 min.

[b]The blocking MAb is an unrelated MAb selected for its ability to bind to RAM-G1.

[c]S_1 is the ratio of conjugate binding signal to the first MAb binding signal. Since the conjugate and MAb have practically the same MW, the ratio represents the relative amount bound.

[d]S_2 is the ratio of the second MAb binding signal to the conjugate binding signal. Here again the ratio represents the relative amount bound.

DISCUSSION

To develop early detection methods for anti-HIV antibodies, the use of peptides mimicking an immunodominant and conserved epitope of the envelope Gp41 protein appears to be a strategy of choice. An assay format using a peptide (39GC) corresponding to the conserved fragment aa 584–609 of Gp41 (HIV-1, BRU isolate) and coupled to alkaline phosphatase was studied to develop an immunoassay for anti-Gp41 on the new ACCESS® instrument (De Kerdaniel *et al.*, 1993). We added a spacer CGG sequence to the N-terminus of the peptide to allow its coupling to alkaline phosphatase. We also used S-ethylcarbamoyl protected cysteines in place of C_{603} and C_{609}. As for other peptides (Storey *et al.*, 1972), we showed that this modification was easily reversed by mild alkaline treatment, leading to a peptide with native conformation.

As suggested by different authors (Wang *et al.*, 1986; Gallaher *et al.*, 1989; Lacroix *et al.*, 1989), at least one epitope of this peptide sequence is located in the internal loop region, which is consistent with our results for MAb 9-11. The binding of MAbs 41-1 and 9-11 to 39GC immobilized on a BIAcore™ sensor chip through its terminal cysteine showed the presence of two epitopes. The epitope recognized by MAb 41-1 does not involve

the internal cysteines as shown by MAb 41-1 binding to SH-protected 39GC. On the other hand, the epitope recognized by MAb 9-11 is masked if the cysteines are protected with S-ethylcarbamoyl groups; binding only occurs after deprotection. These two epitopes are independent, suggesting that they are relatively spaced.

These results were confirmed by kinetic measurements. Since the recognition of peptide 39GC by MAb 9-11 was absolutely dependent on cysteine protecting group cleavage, the binding kinetics were determined after alkaline treatment of the immobilized peptide. Although MAb 41-1 recognizes an epitope near the N-terminal region of the peptide, the equilibrium constants for the binding of this MAb to the two forms of the peptide were different; this difference was found to be due to a dramatic decrease of the dissociation rate constant when the peptide was deprotected. It is well established that conformational constraints can lower the dissociation of a complex by maintaining the most favorable antigen conformation but they can also lower the association reaction because the change of conformation necessary to reach the transient complex state has become more difficult (Pecht and Lancet, 1977).

We had also to show that the epitope in the internal loop was still accessible on the peptide–enzyme conjugate, since the conjugation reaction might influence the state of the internal loop region. We chose a conjugation strategy based on the use of an N-terminal cysteine, purposely added to the peptide sequence for covalent linkage to the enzyme as well as to the sensor chip, and post-coupling internal cysteine deprotection to recover the native conformational loop. This choice had the advantage of locating the peptide–enzyme bond far from the epitope located near the loop. Using the BIAcore™ technique, we were able to determine the parameters for the binding of the conjugate in solution to the MAbs immobilized on the sensor surface through an anti-mouse antibody (RAM-G1); this protocol is similar to the format of the immunoassay for anti-HIV-1 detection in which the antibody is bound to magnetic beads through protein A (De Kerdaniel *et al.*, 1993). Binding experiments showed that roughly one peptide was linked per enzyme molecule. This amount of peptide per enzyme is consistent with the minimum value of 1.5 active groups incorporated after alkaline phosphatase activation. Epitope mapping experiments showed that both 39GC epitopes were present, demonstrating the efficiency of the post synthesis treatment to obtain the native peptide conformation.

The determination of the kinetic rate constants confirmed the high affinity binding of MAb 9-11 to the internal deprotected cysteine region. The equilibrium constant, as well as the association and dissociation rate constants, are very close to those determined for the binding of MAb 9-11 to 39GC immobilized on the dextran surface and treated at pH 9.7. On the contrary, the equilibrium constant for MAb 41-1 binding to the conjugate is intermediate between those calculated for the peptide treated or not treated at pH 9.7. It should be observed that, whereas the dissociation rate constant for the conjugate is also intermediate, the association rate constant is slightly higher than the two rate constants obtained for the surface-linked peptide. These results suggest that once again the flexibility of the peptide is involved, but in this case, the effect due to peptide conformation constraint brought about by cysteine deprotection and loop formation might be balanced by the fact that the peptide is linked to a more flexible support, namely an enzyme in solution.

In conclusion, we showed that the two MAbs 9-11 and 41-1 are specific for two independent epitopes of the major HIV-1 envelope Gp41 peptide fragment aa 584–609. These MAbs appeared to be very efficient tools to characterize the influence of peptide conformational change induced by protection of the two key internal cysteines and by conjugation to soluble alkaline phosphatase. The epitope recognized by MAb 9-11 involves a cysteine residue; it appeared to be independent of the support since it reacted similarly when the peptide was either covalently attached to the dextran matrix or conjugated to the enzyme in solution, provided that formation of the internal loop was possible. Recognition by MAb 41-1 of its epitope appeared sensitive to both the internal peptide conformation and the effect of support presentation possibly because of changes in peptide flexibility.

Taken together, these results show that this type of peptide and the described strategy for conjugate synthesis leading to correct epitope presentation can be used in a novel anti-HIV antibody assay based on total immunocapture on protein A solid phase and specific revelation by a phosphatase alkaline antigen conjugate. A highly sensitive and fast one-step fully automated immunoassay on the new ACCESS® instrument has been developed (De Kerdaniel *et al.*, 1993).

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